Journal of Clinical Laboratory Analysis 11:267–286 (1997)

More Reliable Diagnosis of Infection With Human Immunodeficiency Virus Type 1 (HIV-1) by Detection of Antibody IgGs to *pol* and *gag* Proteins of HIV-1 and p24 Antigen of HIV-1 in Urine, Saliva, and/or Serum With Highly Sensitive and Specific Enzyme Immunoassay (Immune Complex Transfer Enzyme Immunoassay): A Review

Seiichi Hashida, Kazuya Hashinaka, Setsuko Ishikawa, and Eiji Ishikawa*

Department of Biochemistry, Miyazaki Medical College, Miyazaki, Japan

Ultrasensitive enzyme immunoassays (immune complex transfer enzyme immunoassays) were developed for antibody IgGs to HIV-1 using recombinant reverse transcriptase (rRT), p17 (rp17), and p24 (rp24) as antigens. Antibody IgGs were reacted with 2,4-dinitrophenyl-recombinant antigens and recombinant antigen-β-D-galactosidase conjugates, and the immune complexes formed, comprising the three components, were trapped onto polystyrene beads coated with (anti-2,4-dinitrophenyl group) IgG. After washing, the immune complexes were eluted from the polystyrene beads with excess of εN-2,4-dinitrophenyl-L-lysine and were transferred to clean polystyrene beads coated with (antihuman IgG γ-chain) IgG. β-D-Galactosidase activity bound to the last polystyrene beads was assayed by fluorometry. By transfer of the immune complexes from one solid phase to another, the nonspecific binding of the β-D-galactosidase conjugates was minimized and the sensitivity was markedly improved. The immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 as antigens were 300–1,000-fold, 1,000–3,000-fold, and 30–100-fold, respectively, more sensitive than Western blotting for the corresponding antigens and 10–300-fold more sensitive than a conventional ELISA and a gelatin particle agglutination test. For urine (100 μl), whole saliva (1 μl), and serum (1 μl) samples, the sensitivity and specificity of the immune complex transfer enzyme immunoassay using rRT as antigen were both 100%. However, for urine samples in which the specific activities of antibody IgG to RT, p17, and p24 were much lower than those in serum samples probably due to degradation by the kidney, a longer assay of bound β-D-galactosidase activity or/and a concentration process for urine was required. The use of more than 1 μl of whole saliva was recommended for reliable diagnosis of the infections, whereas 1μl of serum was sufficient for the purpose. The positivity with rRT as antigen could be confirmed by demonstration of antibody IgGs to p17 and p24 in most of the urine, whole saliva, and serum samples. In HIV-1 seroconversion serum panels, antibody IgG to p17 was detected as early as or even earlier than antibodies to HIV-1 by a conventional ELISA or/and a gelatin particle agglutination test, whereas antibody IgGs to RT and p24 were detected as early as or later than antibody IgG to p17. Thus the uses of rRT and rp17 as antigens were advantageous over that of the other antigens for randomly collected serum samples probably long after the infection and serum samples at early stages of the infection, respectively. On the basis of these results and other reports, the immune complex transfer enzyme immunoassay was developed for simultaneous detection of p24 antigen and antibody IgGs to RT and p17 in a single assay tube, and the window period (8 weeks, although widely variable), during which diagnosis of HIV-1 infection is not possible due to the absence of detectable antibodies to HIV-1, was shortened by 2 weeks. As a result, the simultaneous detection made possible not only as early diagnosis as that by detection of p24 antigen, but also as reliable diagnosis as that by detection of antibodies to HIV-1. Finally, the immune complex transfer enzyme immunoassay has been recently improved so as to be performed within shorter periods of time (2–3 hr) with higher sensitivity, and testing many samples has become easy. J. Clin. Lab. Anal. 11:267–286, 1997. © 1997 Wiley-Liss, Inc.

*Correspondence to: Dr. Eiji Ishikawa, Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan.

Received 3 January 1997; accepted 6 February 1997.

Key words: human immunodeficiency virus type 1; antigen; antibody; reverse transcriptase; p17; p24; enzyme immunoassay; β-o-galactosidase

INTRODUCTION: REVIEW OF ENZYME IMMUNOASSAY APPLICATIONS

For diagnosis of infection with human immunodeficiency virus type 1 (HIV-1), antibodies to HIV-1 in serum, plasma and whole blood samples have been detected by various methods such as enzyme-linked immunosorbent assay (ELISA) and agglutination test with latexes, red cells, and gelatin particles, and Western blotting has been used as a confirmatory test (1). However, some drawbacks have been noted. There is a window period after the infection during which no antibodies to HIV-1 can be detected (2–7). There are significant numbers of indeterminate results due to low sensitivity and specificity of Western blotting as a confirmatory test (2,8–10). Blood samples should be collected and handled with due caution to avoid infections, not only with HIV but also with other pathogens such as hepatitis B and C viruses (11). In order to minimize the possibility of infections and to reduce the costs of tests, attempts have been made to diagnose HIV-1 infection by detecting antibodies to HIV-1 in urine and saliva (11–14). However, the sensitivity and specificity of currently available methods are not satisfactory (12–14).

This article reviews the application of ultrasensitive and highly specific enzyme immunoassays (immune complex transfer enzyme immunoassays) for antibody IgGs to *pol* (reverse transcriptase (RT)) and *gag* (p17 and p24) proteins of HIV-1 and for p24 antigen of HIV-1, which significantly overcome the above drawbacks of conventional methods in diagnosis of HIV-1 infection (12,15–24).

CONCENTRATION OF IgG IN URINE, SALIVA, AND SERUM

In healthy and HIV-1 seronegative subjects aged 7–87 yr, the concentrations of IgG in urine and whole saliva samples measured by two-site enzyme immunoassay were 3.8 ± 4.7 (SD) μg/ml (range, 0.05–41 μg/ml; n=279) (12), and 35 ± 36 (SD) μg/ml (range, 0.7–187 μg/ml; n=76) (18), respectively. These were 4,000-fold and 400-fold, respectively, lower than those in serum samples (15.2 ± 6.3 (SD) mg/ml; range, 3.8–36 mg/ml; n=105) (Fig. 1) (12). There was no significant difference between the sexes of IgG concentration in urine, whole saliva, and serum samples. Urine samples were collected randomly without controlling physical positions. However, it is recommended that urine samples be collected after some physical activities, since the concentration of IgG in urine samples tends to be higher in the upright position or during exercises than in a lying position. Whole saliva samples were collected by simple spitting without using any devices, although some devices have been described to collect saliva

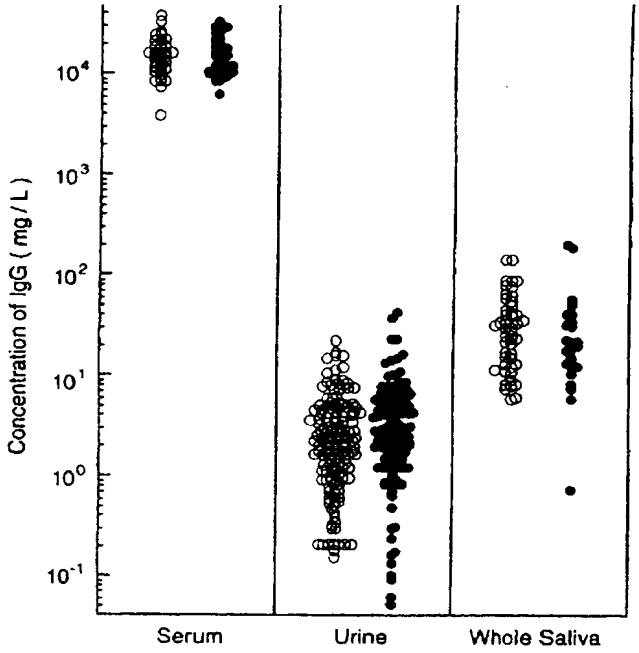

Fig. 1. Concentration of IgG in serum, urine, and whole saliva from healthy HIV-1 seronegative subjects. Urine samples were collected without controlling physical positions. Whole saliva samples were collected by simple spitting without using any devices. The concentration of IgG was measured by two-site enzyme immunoassay. Open and closed circles indicate values for males aged 7–85 yr and females aged 8–87 yr, respectively.

samples containing high concentrations of IgG (13). In HIV-1-infected subjects, the concentration of IgG in urine (12) (Fig. 2) and whole saliva (18) (Fig. 3) samples tended to be enhanced, but only slightly. Therefore, an ultrasensitive method for the detection of antibody IgG to HIV-1 in urine and whole saliva samples is required.

IMMUNE COMPLEX TRANSFER ENZYME IMMUNOASSAY FOR ANTIBODY IgG TO HIV-1

The principle of the immune complex transfer enzyme immunoassay has been described in detail elsewhere (23). In brief, the immune complex formed by reacting antibody to be detected with labeled antigen is trapped onto solid phase and, after washing the solid phase, is transferred to another clean solid phase to minimize the nonspecific signal for achieving high sensitivity. The most sensitive version among several variations of the immune complex transfer enzyme immunoassay is shown schematically in Figure 4. Antibody to be detected is reacted simultaneously with 2,4-dinitrophenyl-antigen and enzyme-labeled antigen. The immune complex formed, comprising the three components, is trapped

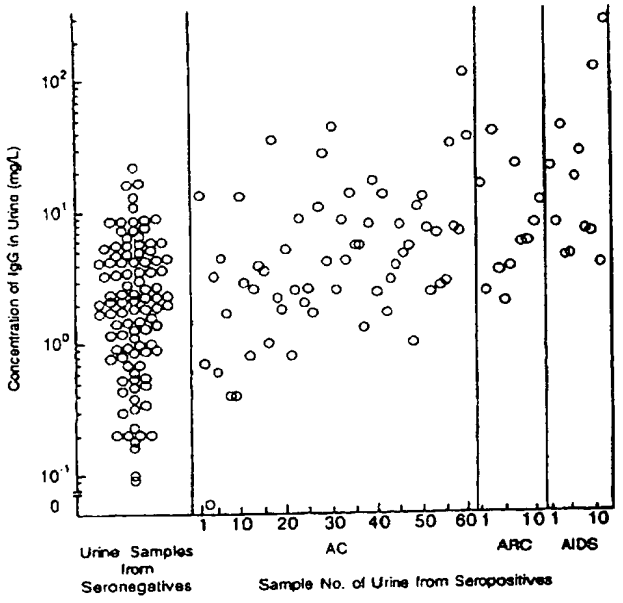

Fig. 2. Concentration of IgG in urine samples from 100 HIV-1 seronegative subjects and 83 HIV-1 seropositive subjects (60 asymptomatic carriers, 11 patients with ARC and 12 patients with AIDS) shown in Figure 6.

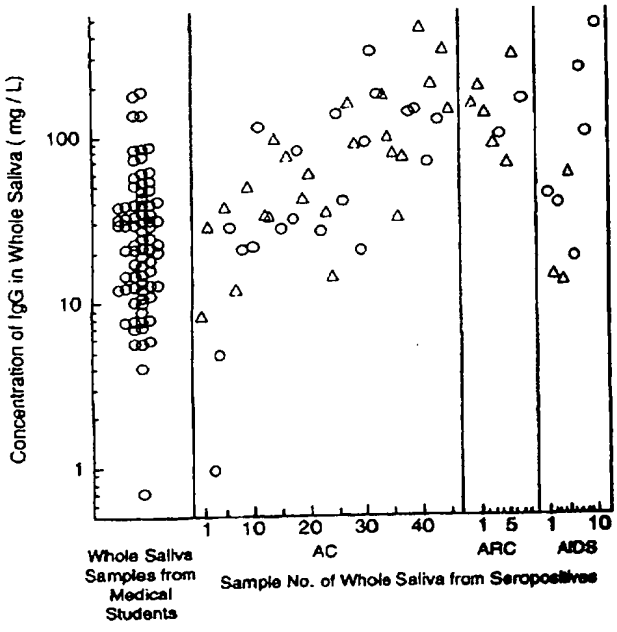

Fig. 3. Concentration of IgG in whole saliva samples from 76 medical students and 61 HIV-1 seropositive subjects (44 asymptomatic carriers, 8 patients with ARC and 9 patients with AIDS) shown in Figure 9. Triangles and circles indicate values for hemophiliacs and nonhemophiliacs, respectively.

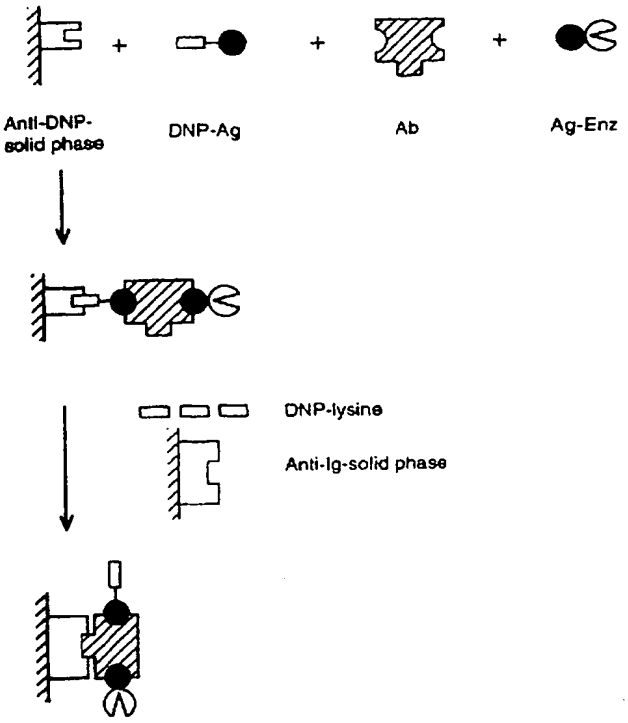

Fig. 4. Immune complex transfer enzyme immunoassay for antibody IgG. DNP: 2,4-dinitrophenyl group. Ag: antigen. Ab: antibody. Enz: enzyme. Ig: immunoglobulin.

onto solid phase coated with (anti-2,4-dinitrophenyl group) IgG. After washing, the immune complex is eluted from the solid phase with excess of εN-2,4-dinitrophenyl-L-lysine and is transferred to clean solid phase coated with anti-immunoglobulin IgG. Finally, the enzyme activity bound to the last solid phase is assayed by fluorometry. By transfer from solid phase to solid phase of the immune complex, the nonspecific signal is reduced to a great extent, and the sensitivity can be improved markedly as compared with those of conventional methods (23). The use of β-D-galactosidase from *Escherichia coli* as label provides 30 times as sensitive immune complex transfer enzyme immunoassay as that of horseradish peroxidase (12,16,24).

Recombinant proteins used as antigens in the immune complex transfer enzyme immunoassay reviewed in this article are recombinant reverse transcriptase (rRT), p17 (rp17), and p24 (rp24), which were produced in *E. coli* transformed with expression plasmids carrying the corresponding cDNAs and were purified as described previously (15,25–27). The recombinant proviral clone used was pNL4-3 (28), which contained DNA from HIV-1 isolates NY5 (GenBank accession number HIVNL43) and LAV (29), and the sequences for rRT, rp17, and rp24 derived from NY5.

By applying the same principle as used for the detection of antibodies, p24 antigen of HIV-1 has been measured with high sensitivity (22).

DIAGNOSIS OF HIV-1 INFECTION WITH URINE

Stability of Antibody IgG to HIV-1 in Urine

Urine samples immediately after collection were mixed with 1/100 volume each of 10 mg/ml bovine serum albumin and 100 mg/ml NaN_3, or 5 mg/ml thimerosal and were stored at -20°C (12,15–17). Under this condition using thimerosal, anti-HIV-1 IgG detectable by the immune complex transfer enzyme immunoassay using rRT as antigen and horseradish peroxidase as label was stable for at least 6 months (Fig. 5) (12,15).

Effect of Urine Volume and pH on Signal

The signal by the immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 as antigens and β-D-galactosidase from *E. coli* as label (the fluorescence intensity for β-D-galactosidase activity bound to solid phase coated with (antihuman IgG γ-chain) IgG) is only slightly influenced by the volume of urine up to 100 μl (12,15–17), but varies depending on pH of urine. The maximal signals for HIV-1 seropositive subjects were observed at pH 7.5–8.0 with rRT (12,15,16) and rp17 (12,15) as antigens, and at pH 5.0 with rp24 as antigen (12,17). The signals were lowered by 21–30% at pH 5.0–5.5 with rRT (12,15,16) and rp17 (12,15) as compared with the maximal signal, and by 29% at pH 8.0 with rp24 (12,17). The nonspecific signals for HIV-1 seronegative subjects were slightly enhanced at lower pH with rRT (12,15,16) and rp17(12,15), and at higher pH with rp24 (12,17). From these results, appropriate adjustment of pH of urine samples is recommended for effective detection of antibody IgG to HIV-1, since pH of urine samples varied from 5.0 through 8.0.

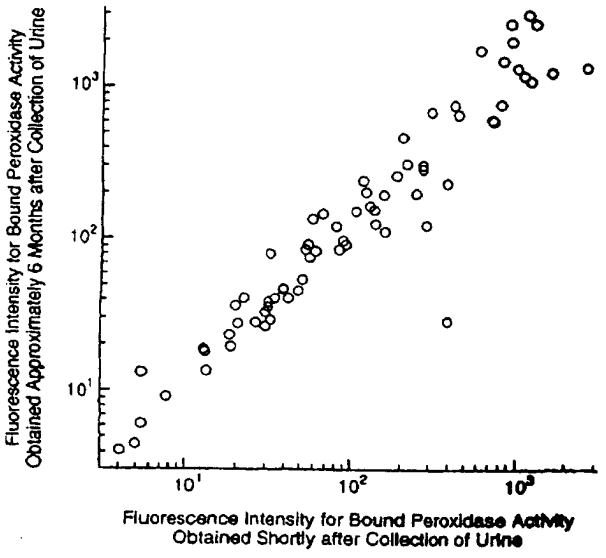

Fig. 5. Stability of antibody IgG to HIV-1 in urine. Urine samples were collected from 71 HIV-1 seropositive subjects and were stored at -20°C after addition of 1/100 volume each of 10 mg/ml bovine serum albumin and 5 mg/ml thimerosal. Antibody IgG to HIV-1 in urine was measured by the immune complex transfer enzyme immunoassay using rRT as antigen and horseradish peroxidase as label shortly after collection (abscissa) and 6 months after the storage (ordinate).

Sensitivity and Specificity of Immune Complex Transfer Enzyme Immunoassay

Eighty-three urine samples from HIV-1 seropositive subjects—60 asymptomatic carriers, 11 patients with AIDS-related complex (ARC), and 12 patients with AIDS—and 100 urine samples from HIV-1 seronegative subjects were tested by the immune complex transfer enzyme immunoassay using rRT as antigen and β-D-galactosidase from *E. coli* as label (12,15,16). The volume of urine samples used was 100 μl. The ratios of the lowest signals for the asymptomatic carriers and the patients with ARC and AIDS to the highest signal for the seronegative subjects were 8.2, 11, and 7.2, respectively, when bound β-D-galactosidase activity was assayed for 2.5 hr (Table 1). By a longer assay (25 hr) of bound β-D-galactosidase activity, the ratios were enhanced to 14, 29, and 10, respectively (Fig. 6A, Table 1). The ratios were also significantly enhanced by using 10-fold concentrated urine samples, which can by prepared by 20 min centrifugation in a microconcentrator (Fig. 7) and by the combined use of rRT, rp17, and rp24, since signals with rRT as antigen were lower than those with rp17 or/and rp24 as antigens in some HIV-1 seropositive subjects (Table 2).

Thus the sensitivity and specificity of the immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 in urine using rRT as antigen and β-D-galactosidase from *E. coli* as label were both 100% (sensitivity: 60/60 for asymptomatic carriers, 11/11 for patients with ARC, and 12/12 for patients with AIDS and specificity: 100/100) with no indeterminate results using 100 μl of unconcentrated urine samples (Fig. 6) and were expected to be both 100% even for a larger number of samples by using both 10-fold concentrated urine samples and the three antigens (rRT, rp17 and rp24) (Table 2). Furthermore, the positivity could be confirmed by separately demonstrating antibody IgG to each of rRT, rp17 (12,15,16), and rp24 (12,17) in most cases (Fig. 6). However, it is recommended to measure IgG in each urine sample and not to make negative diagnosis for urine samples containing < 1 μg/ml of IgG (Fig. 2).

Sensitivity and Specificity of Conventional Methods

The sensitivity and specificity for urine samples from the above seropositive and seronegative subjects (Fig. 6) of the con-

TABLE 1. Lowest Cutoff Index (Ratio of the Lowest Signal for HIV-1 Seropositive Subjects to the Highest Nonspecific Signal for HIV-1 Seronegative Subjects) by Immune Complex Transfer Enzyme Immunoassay Using rRT as Antigen

Sample (sample volume used)	Assay time for bound β-D-galactosidase activity (hr)	Lowest cutoff index		
		AC[a]	ARC[b]	AIDS[c]
Urine (unconcentrated, 100 μl)	2.5	8.2	11	7.2
	25	14	29	10
Whole saliva (1 μl)	2.5	38	78	3.0
Serum (1 μl)	2.5	22,400	26,400	2,080
Serum (10 μl)	2.5	56,000	66,000	5,200

[a]Asymptomatic carriers.
[b]Patients with AIDS-related complex.
[c]Patients with AIDS.

ventional ELISA (Abbott HTLV-III EIA with recombinant gp 120, gp41, p24, p17, and p15 as antigens, Abbott Lab, North Chicago, IL) and the gelatin particle agglutination test (Serodia-HIV with lysate of HIV-1 as antigen, Fujirebio, Tokyo, Japan) were 77–89% and 97–99%, respectively (12,15). With approximately 10-fold concentrated urine samples, the sensitivity was slightly improved, but the specificity was lowered to 57–76% due to enhanced nonspecific signals (12,15).

In other laboratories, antibodies to HIV-1 in urine samples were detected by the conventional ELISA (30–32), IgG antibody-capture, enzyme—linked immunosorbent assay (GACELISA) (33–35) and IgG antibody-capture particle adherence test (GACPAT) (33,34). The sensitivity and specificity of the conventional ELISA performed using various commercial kits were 93.0–100% and 92.3–100%, respectively (30,31). In only one report, both the sensitivity and specificity were 100%, but the ratio of signal to noise was not improved by concentration of urine samples (32). The sensitivity and specificity of GACELISA were 88.1–99.4% and 97.9–100%, respectively (33–35). Those of GACPAT were 95.2–100% and 97.9–99.6%, respectively (33,34). In the last two methods, the sensitivity would not be improved by using concentrated urine samples, as long as the level of IgG in urine samples reached the low level needed to saturate the assay anti-IgG binding sites (33).

Correlation of Levels of Antibody IgG to HIV-1 in Urine to Those in Serum

Twenty-five paired urine (Y) and serum (X) samples from 17 asymptomatic carriers, 3 patients with ARC, and 5 patients with AIDS were tested by the immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 as antigens and β-D-galactosidase from *E. coli* as label (Fig. 8). When signals were corrected with the sample volume alone, the regression equations and the correlation coefficients were log(Y)=0.83 log(X)-3.6 and r = 0.45 with rRT as antigen, log(Y)=0.65 log(X)-2.4 and r = 0.63 with rp17 and log(Y)=0.77 log(X)-2.9 and r = 0.85 with rp24. These were improved by correction with the amount of IgG to log(Y)=1.0 log(X)-1.6 and r = 0.66 with rRT, log(Y)=0.73 log(X)-0.64 and r=0.79 with rp17 and log(Y)=0.82 log(X)-0.51 and r = 0.87 with rp24. The specific activity of antibody IgG to RT of HIV-1 (the signal corrected by the amount of IgG) in urine samples was 3.9–34-fold lower in 56% of the 25 samples than that in serum samples, 50–115-fold lower in 32%, and 213–979-fold lower in 12%. The specific activity of antibody IgG to p17 and p24 was also lower in urine samples than in serum samples, although to less degrees. These results suggested that antibody IgGs to HIV-1 were inactivated to various degrees in the kidney, but not drastically in urine samples collected, since antibody IgG to HIV-1 RT was stable at −20°C for 6 months as described above and after several freezing-thawings.

DIAGNOSIS OF HIV-1 INFECTION WITH WHOLE SALIVA

Stability of Antibody IgG to HIV-1 in Whole Saliva

Whole saliva samples were collected by simply spitting without using any devices and were frozen at -20°C immediately after collection. The frozen samples were thawed and centrifuged at 10,000 x g for 10 min to remove precipitates before use, or were thawed and centrifuged to store the supernatant at -20°C until use. Under this condition, antibody IgG to HIV-1 detectable by the immune complex transfer enzyme immunoassay using rRT as antigen and β-D-galactosidase from *E. coli* as label was stable for at least 3 1/2 months (18).

Effect of Whole Saliva Volume on Signal

When the volume of whole saliva was increased up to 100 μl/assay, the signal by the immune complex transfer enzyme immunoassay using rRT as antigen and β-D-galactosidase from *E. coli* as label was linearly increased for HIV-1 seropositive subjects, but only slightly increased for HIV-1 seronegative subjects (18).

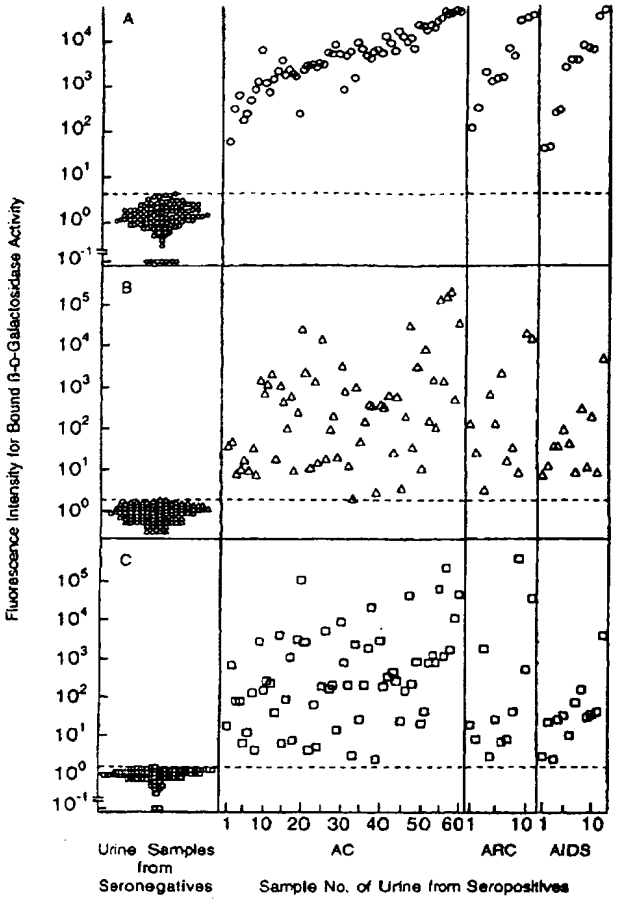

Fig. 6. Detection of antibody IgG to HIV-1 in unconcentrated urine samples. Urine samples from 83 HIV-1 seropositive subjects (60 asymptomatic carriers, 11 patients with ARC, and 12 patients with AIDS) and 100 HIV-1 seronegative subjects were tested by the immune complex transfer enzyme immunoassays using rRT (A, circles), rp17 (B, triangles), and rp24 (C, squares) as antigens and β-D-galactosidase from *E. coli* as label. The volume of urine samples used was 100 μl. Bound β-D-galactosidase activity was assayed at 30°C for 25hr. The broken lines indicate the highest signals for the seronegative subjects. AC: asymptomatic carriers. ARC: patients with AIDS-related complex. AIDS: patients with AIDS.

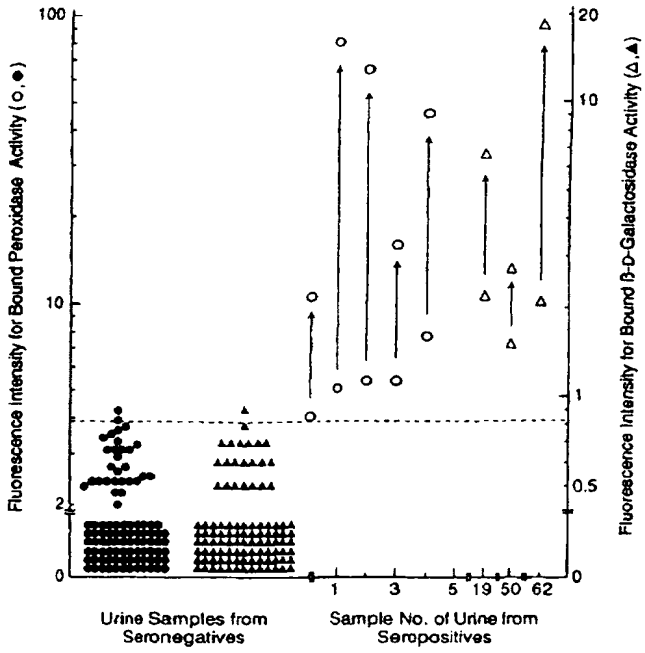

Fig. 7. Confirmation of the positivity and negativity by concentration of urine samples. Urine samples, which gave low signals by the immune complex transfer enzyme immunoassays using rRT (circles) and rp17 (triangles) as antigens and horseradish peroxidase and *E. coli* β-D-galactosidase, respectively, as labels were concentrated 10-fold and tested by the same enzyme immunoassays. The volumes of unconcentrated and concentrated urine samples used were both 100 μl. Open and closed symbols indicate signals for HIV-1 seropositive and seronegative subjects, respectively. Arrows indicate changes by the concentration. The broken line indicates the highest signals for unconcentrated urine samples of seronegative subjects.

Sensitivity and Specificity of Immune Complex Transfer Enzyme Immunoassay

Whole saliva samples were collected from 63 HIV-1 seropositive subjects (45 asymptomatic seropositives aged 17–47 yr, 8 patients with ARC aged 10–52 yr, and 10 patients with AIDS aged 21–49 yr) and 76 medical students (50 males aged 19–58 yr and 26 females aged 19–26 yr) and were tested

TABLE 2. Signal (Fluorescence Intensity for Bound β-D-Galactosidase Activity) by Immune Complex Transfer Enzyme Immunoassay Using Each of rRT, rp17, and rp24 and a Mixture of Three Antigens[a]

Urine sample	Signal with conjugates of			
	rRT	rp17	rp24	rRT, rp17, and rp24
Seronegatives (n=3)	1.1–1.2	0.2–1.0	0.7–0.8	0.9–1.0
Seropositives				
1	30	35	255	232
2	106	293	11	379

[a]Amount of each recombinant protein conjugate was 100 fmol/assay for separate uses and 30 fmol/assay for the combined use. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr.

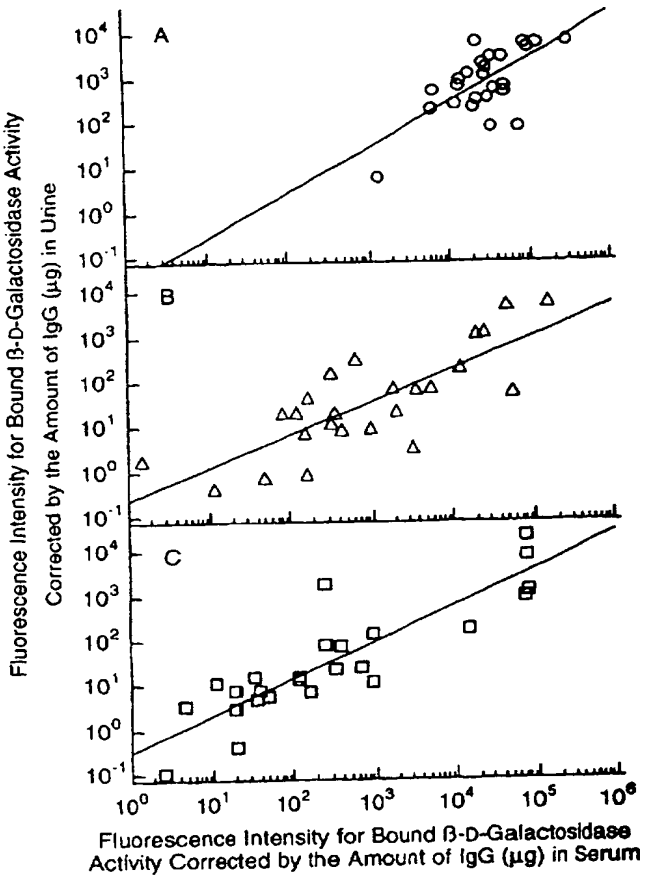

Fig. 8. Correlation between levels of antibody IgG to HIV-1 in urine (Y) and serum (X) samples. Twenty-five paired urine and serum samples from 17 asymptomatic carriers, 3 patients with ARC, and 5 patients with AIDS were tested by the immune complex transfer enzyme immunoassays using rRT(A), rp17(B), and rp24(C) as antigens, and fluorescence intensities for bound β-D-galactosidase activity were corrected by the amount of IgG. The regression equations and correlation coefficients are log(Y)=1.0 log (X)-1.6 and r=0.66 with rRT as antigen, log(Y)=0.73 log (X)-0.64 and r=0.79 with rp17 as antigen and log(Y)=0.82 log (X)-0.51 and r=0.87 with rp24 as antigen.

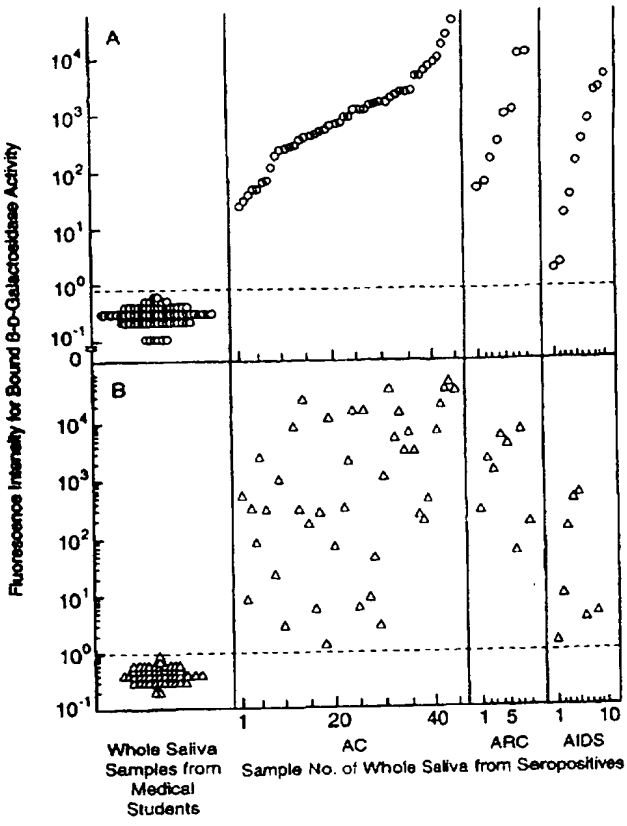

Fig. 9. Detection of antibody IgG to HIV-1 in whole saliva samples. One μl (A, open circles) and 20 μl (B, open triangles) of whole saliva samples from 63 HIV-1 seropositive subjects (45 asymptomatic carriers, 8 patients with ARC, and 10 patients with AIDS) and 76 medical students were tested by the immune complex transfer enzyme immunoassays using rRT (A, circles) and rp17 (B, triangles) as antigens and β-D-galactosidase from *E. coli* as label. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr. The broken lines indicates the highest signals for the medical students.

by the immune complex transfer enzyme immunoassay using rRT as antigen and β-D-galactosidase from *E. coli* as label (Fig. 9) (18). Using as little as 1 μl of whole saliva samples, the ratios of the lowest signals for the asymptomatic carriers and the patients with ARC and AIDS to the highest signal for the medical students were 38, 78, and 3, respectively.

When the volume of whole saliva for test was increased up to 100 μl, signals for HIV-1 seropositive subjects increased proportionally, whereas signals for the medical students increased only slightly (18). Therefore, whole saliva samples containing extremely low levels of antibody IgG to RT of HIV-1, even 2,000-fold lower than the lowest level among the asymptomatic carriers and the patients with ARC and 150-fold lower than the lowest level among the patients with AIDS, were considered to be discriminated from those of HIV-1 seronegative subjects. Positive results with rRT as antigen can be confirmed by demonstrating antibody IgG to rp17 and probably rp24 in most of whole saliva samples. Although 34 subjects out of the 63 seropositives were hemophiliacs, the concentrations of IgG in whole saliva samples from the hemophiliacs and the nonhemophiliacs were not very different, indicating that the above results were not due to bleeding in the oral cavity of the hemophiliacs, if any (Fig. 3) (18).

Thus the sensitivity and specificity of the immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 in whole saliva samples using rRT as antigen and β-D-galactosidase from *E. coli* as label were both 100% (sensitivity: 45/45 for asymptomatic carriers, 8/8 for patients with ARC and 10/10 for patients with AIDS and specific-

ity: 76/76) with no indeterminate results using as little as one microliter of whole saliva samples (Fig. 9) and were expected to remain both 100% even for a larger number of samples by increasing the volume of whole saliva samples up to 100 μl, if necessary. However, it is recommended to measure IgG in each whole saliva sample and not to make negative diagnosis for whole saliva samples containing <10 μg/ml (Fig. 3).

Sensitivity and Specificity of Conventional Methods

In other laboratories, antibody IgG to HIV-1 in saliva samples has been detected by different methods. By the conventional enzyme-linked immunosorbent assay (ELISA), the sensitivities and specificities were 88.0–99.5% and 99.5–100%, respectively (36–41), and signals for many of seropositive subjects were close to those for seronegative subjects even using gingival crevicular transudate samples (38,41), containing much higher concentrations of IgG than whole saliva samples (42). By the IgG antibody capture radioimmunoassay (GACRIA) (40,43) and the IgG antibody capture enzyme-linked immunosorbent assay (GACELISA) (34, 35,41,43,44), the sensitivities and specificities were 96.9–100% and 99.8–100%, respectively. Using as much as 50–100 μl of saliva samples, however, the lowest signal among seropositive subjects was almost equal to or up to only 4-fold higher than the highest signal among seronegative subjects (35,40,41,43,44), and the number of seropositive subjects and seronegative subjects tested was limited (32–55 and 10–55, respectively) (35,41,43,44), although 196 and 460, respectively, in one report using GACRIA (40). Therefore, the sensitivity or/and specificity may be lowered for a larger number of samples.

Correlation of Levels of Antibody IgG to HIV-1 in Whole Saliva to Those in Serum

Paired samples of whole saliva (Y) and serum (X) from 30 to 34 HIV-1 seropositive subjects (22–24 asymptomatic carriers, 4 patients with ARC, and 4–6 patients with AIDS) were tested by the immune complex transfer enzyme immunoassays using rRT and rp17 as antigens and β-D-galactosidase from *E. coli* as label (Fig. 10) (18). The regression equations and the correlation coefficients were $\log(Y)=1.2\log(X)-3.6$ and $r = 0.81$ with rRT as antigen and $\log(Y)=0.87\log(X)-2.0$ and $r= 0.91$ with rp17 as antigen, when signals were corrected by the sample volume alone. These were improved to $\log(Y)= 0.98\log(X)-0.19$ and $r = 0.92$ with rRT as antigen and $\log(Y)=0.78\log(X)+0.55$ and $r = 0.95$ with rp17 as antigen, when signals were corrected by the amount of IgG.

Level of Antibody IgA to HIV-1 in Saliva

In saliva samples, the concentration of IgA is higher than that of IgG (23,42). In order to detect not only antibody IgG to HIV-1, but also anti-HIV-1 antibodies of other classes, 2,4-dinitrophenyl-biotinyl-bovine serum albumin-rRT conjugate and streptavidin-coated polystyrene beads were substituted for 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate and (anti-human IgG γ-chain) IgG-coated polystyrene beads, respectively, in the immune complex transfer enzyme immunoassays described above. This substitution makes possible the detection of both IgG antibodies and antibodies of other classes with similar sensitivities (23). Ten whole saliva samples from HIV-1 seropositives were tested, and signals were not significantly increased. Therefore, the concentration of anti-HIV-1 antibodies of other classes may be low in whole saliva samples, if any.

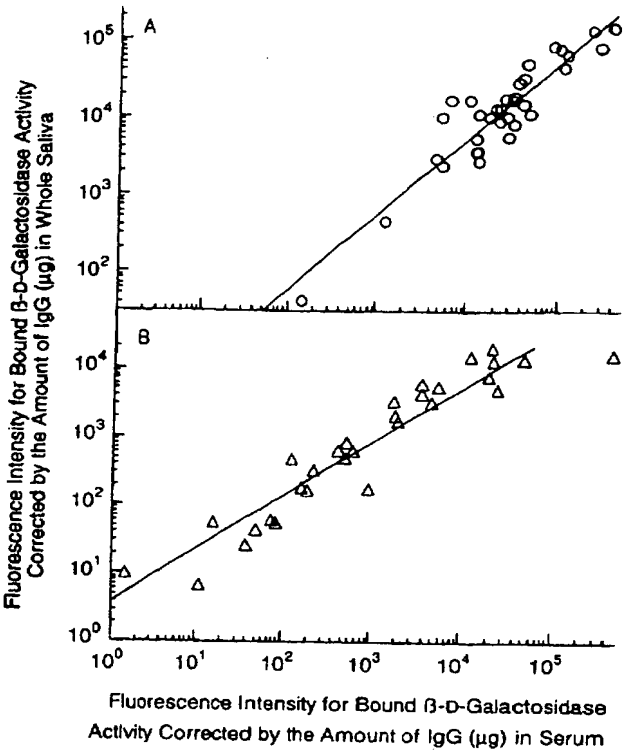

Fig. 10. Correlation between levels of antibody IgG to HIV-1 in whole saliva (Y) and serum (X) samples. A total of 30–34 paired whole saliva and serum samples from 22–24 asymptomatic carriers, 4 patients with ARC, and 4–6 patients with AIDS were tested by the immune complex transfer enzyme immunoassays using rRT (A) and rp17 (B) as antigens, and fluorescence intensities for bound β-D-galactosidase activity were corrected by the amount of IgG. The regression equations and correlation coefficients are $\log(Y)=0.98\log(X)-0.19$ and $r=0.92$ with rRT as antigen and $\log(Y)=0.78\log(X)+0.55$ and $r=0.95$ with rp17 as antigen.

Diagnosis of HIV-1 Infection Using Whole Saliva Dried on Filter Paper

The diagnosis of HIV-1 infection in asymptomatic carriers has been shown to be possible using even dried whole saliva

samples (2-3 μl) on filter paper discs with a diameter of 3 mm as a substitute for frozen ones (45).

Significance of Diagnosis of HIV-1 Infection With Whole Saliva

The diagnosis of HIV-1 infection by the immune complex transfer enzyme immunoassay using whole saliva samples is significant from the following viewpoints (18). Whole saliva can be collected with no invasive procedures, no particular devices, minimal expenses, and minimal possibility of infections with other pathogens. Reproducible results can be obtained using only small volumes of whole saliva samples. This is important, since whole saliva samples collected by simple spitting and subsequent centrifugation is small in volume in some cases, making difficult repeated duplicate assays with larger volumes, although the use of larger volumes appears to be more practical. For the diagnosis of HIV-1 infection, one may only have to send one's whole saliva samples to a facility for test after freezing in a vial, e.g., with only a number for identification. Dentists may also make use of test results with whole saliva rather than serum or gingival crevicular transudate samples (14,38) prior to dental treatments with possible bleeding. The use of whole saliva samples that can be collected with greater ease and lower costs than serum and gingival crevicular transudate samples (14,38) makes the diagnosis of HIV-1 infection easier in developing countries.

DIAGNOSIS OF HIV-1 INFECTION WITH SERUM

Effect of Serum Volume on Signal (Serum Interference)

When increasing volumes of two serum samples from HIV-1 seropositive subjects were tested by the immune complex transfer enzyme immunoassays using rRT, rp17 and rp24 as antigens and β-D-galactosidase from *E. coli* as label, the signal was enhanced almost linearly with up to 5 μl, and only slight interference was observed with 10 μl of serum (19).

Sensitivity of Immune Complex Transfer Enzyme Immunoassay Compared With Those of Other Methods

Serum samples from 79 HIV-1 seropositive subjects (50 asymptomatic carriers, 9 patients with AIDS-related complex (ARC) and 20 patients with AIDS) and 100 HIV-1 seronegative subjects were tested by the immune complex transfer enzyme immunoassays (Fig. 11) (19). The volume of serum samples used was 10 μl. The ratios of the lowest signals with rRT as antigen for the asymptomatic carriers and the patients with ARC and AIDS to the highest signal for the seronegative subjects were 56,000, 66,000, and 5,200, respectively. Even with 1 μl of serum samples, the ratios were 22,400, 26,400, and 2,080, respectively, since the highest signal for the seronegative subjects was lowered by reducing the volume of serum samples used (18). Using 10 μl of serum samples, the ratios with rp17 as antigen for the asymptomatic carriers and the patients with ARC were 680 and 1,200, respectively, and the ratios with rp24 as antigen for the asymptomatic carriers and the patients with ARC were 22 and 89, respectively. In the patients with AIDS, however, one serum sample was negative with rp17 as antigen, and two serum samples were negative with rp24 as antigen.

Thus the sensitivity of the immune complex transfer enzyme immunoassay using each of the three antigens was 100%

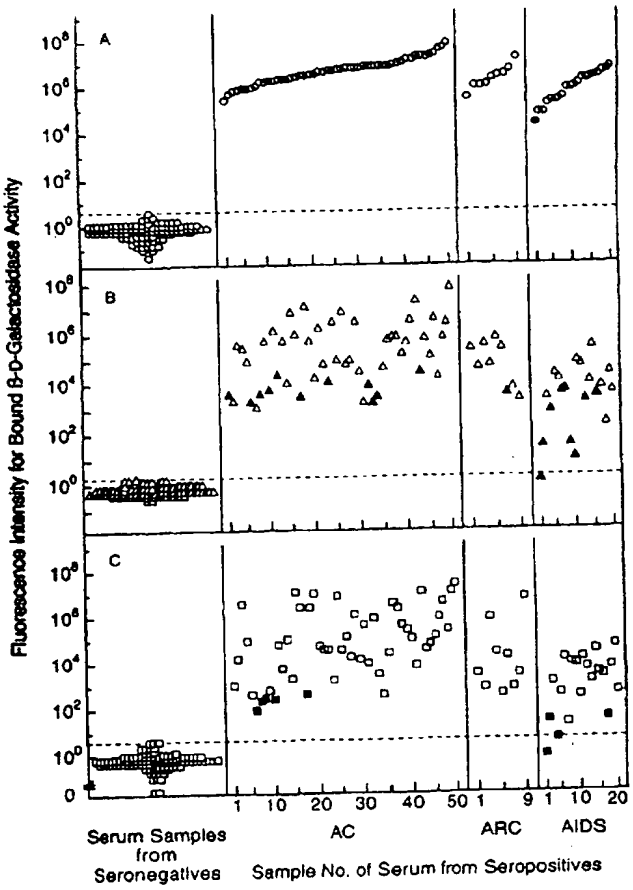

Fig. 11. Detection of antibody IgG to HIV-1 in serum samples. Serum samples (10 μl) from 79 HIV-1 seropositive subjects (50 asymptomatic carriers, 9 patients with ARC, and 20 patients with AIDS) and 100 HIV-1 seronegative subjects were tested by the immune complex transfer enzyme immunoassays using rRT (A, circles), rp17 (B, triangles), and rp24 (C, squares) as antigens and β-D-galactosidase from *E. coli* as label. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr. Open and closed symbols indicate serum samples tested positive and negative, respectively, by Western blotting for the corresponding antigens. The broken lines indicate the highest signals among the 100 seronegative subjects, which were taken as tentative cutoff values as described in Fig. 12.

for the asymptomatic carriers (50/50) and the patients with ARC (9/9). For the patients with AIDS, the sensitivities were 100% with rRT (20/20), 95% with rp17 (19/20) and 90% with rp24 (18/20). By contrast, the sensitivities of Western blotting (Ortho Diagnostic Systems, Raritan, NJ) were lower (Fig. 11). The sensitivities of Western blotting for p66 (one of RT components), p51 (the other component of RT), p17, and p24 bands were 100% (50/50), 100% (50/50), 78% (39/50), and 90% (45/50), respectively, in the asymptomatic carriers, 100% (9/9), 100% (9/9), 89% (8/9), and 100% (9/9), respectively, in the patients with ARC and 95% (19/20), 75% (15/20), 60% (12/20), and 80% (16/20), respectively, in the patients with AIDS (Table 3).

The sensitivities with 10 μl of serum samples of the immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 as antigens and β-D-galactosidase from *E. coli* as label were more directly compared with those of other methods by testing low levels of antibodies to HIV-1. Two serum samples from HIV-1 seropositive subjects were serially diluted with serum from an HIV-1 seronegative subject and were tested by the immune complex transfer enzyme immunoassays, Western blotting (Ortho kit), the conventional ELISA using five recombinant proteins of HIV-1 as antigens (Abbott HTLV-III EIA kit), and the gelatin particle agglutination test using a lysate of HIV-1 as antigen (Serodia-HIV kit, Fujirebio, Tokyo, Japan) (Fig. 12) (19). The maximal dilutions of the serum samples to show the positivity were compared. Tentative cutoff values in the immune complex transfer enzyme immunoassays and the conventional ELISA were the highest signals among 100 HIV-1 seronegative subjects and 75 HIV-1 seronegative subjects, respectively. The immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 were 300–1,000-fold, 1,000–3,000-fold, and 30–100-fold, respectively, more sensitive than Western blotting for the corresponding antigens (p66 as one component of RT, p17, and p24) and 10–300-fold more sensitive than the conventional ELISA and the gelatin particle agglutination test, as far as the serum samples tested were concerned. Western blotting for the other component of RT, p51, was less sensitive than that for p66. Similar results were obtained with Sanofi kit.

Specificity of Immune Complex Transfer Enzyme Immunoassay Compared With That of Western Blotting

The specificity of the immune complex transfer enzyme immunoassay using each of the three antigens (rRT, rp17, and rp24) was 100% (100/100) as shown in Figure 11, but was further tested as follows.

Fifty serum samples were collected from healthy subjects with low risk for HIV infection and tested by the conventional ELISA, the gelatin particle agglutination test, and Western blotting using two commercial kits (Ortho Diagnostic Systems and Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) (Table 4) (19). All the serum samples were negative by both the conventional ELISA and the gelatin particle agglutination test. Fourteen serum samples (Serum Nos. 1-14) out of the 50 showed one or two positive bands with only one of the two kits for Western blotting, but not with both kits. Namely, no positive bands for these 14 serum samples with one kit were confirmed with the other. Therefore, positive bands by Western blotting for these 14 serum samples appeared to be false. By the immune complex transfer enzyme immunoassay using each of rRT, rp17, and rp24, all these 50 sera were negative. Thus the specificities of the immune complex transfer enzyme immunoassays and Western blotting were 100% (50/50) and 76% (38/50)—96% (48/50), respectively.

Eighty-five hundreds serum samples from nonhemophiliacs, who visited an institute for HIV test, were also used for the specificity test (Table 4) (19). Fifty-five serum samples out of the 8,500 were positive by conventional ELISA. Thirty serum samples out of the 55 were positive with two or more positive bands for gp120/160, gp41, and p24 by Western blotting (Ortho Diagnostic Systems), and 19 serum samples out

TABLE 3. Sensitivity of Immune Complex Transfer Enzyme Immunoassay and Western Blotting for Serum Samples From HIV-1 Seropositive Subjects

Method	Antigen	Sensitivity for serum samples from patients		
		AC n=50	ARC n=9	AIDS n=20
		%	%	%
Immune complex transfer enzyme immunoassay	rRT	100	100	100
	rp17	100	100	95
	rp24	100	100	90
Western blotting	p66	100	100	95
	p51	100	100	75
	p17	78	89	60
	p24	90	100	80

Bound β-D-galactosidase activity in the immune complex transfer enzyme immunoassays were assayed at 30°C for 2.5 hr.

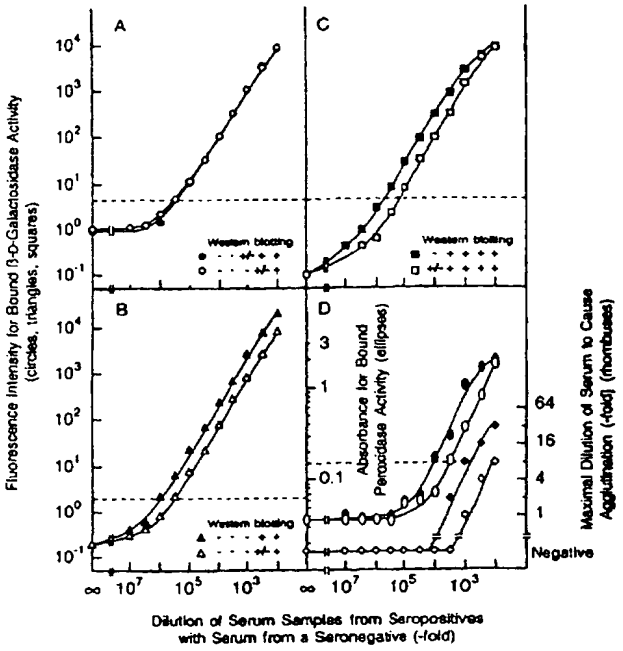

Fig. 12. Dilution curves of two serum samples from HIV-1 seropositive subjects by the immune complex transfer enzyme immunoassays, compared with results by other methods. Two serum samples from HIV-1 seropositive subjects were serially diluted with serum from an HIV-1 seronegative subject and were tested by the immune complex transfer enzyme immunoassays using rRT (A, circles), rp17 (B, triangles), and rp24 (C, squares) of HIV-1 as antigens, β-D-galactosidase from *E. coli* as label, the conventional ELISA (Abbott 2nd generation kit) (D, ellipses), the gelatin particle agglutination test (Fujirebio kit) (D, rhombuses) and Western blotting (Ortho kit). Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr. Open and closed symbols indicate the two different serum samples. The positivity and negativity by Western blotting for the corresponding antigens (p66 as one component of RT, p17 and p24) were expressed as (+) and (−), respectively. The broken lines indicate tentative cutoff values, which are the highest signals among 100 HIV-1 seronegative subjects by the immune complex transfer enzyme immunoassays (4.4 with rRT, 1.9 with rp17, and 4.3 with rp24) and among 75 HIV-1 seronegative subjects by the conventional ELISA (0.15).

of the 55 showed no positive bands with any of two commercial kits (Ortho Diagnostic Systems and Sanofi Diagnostics Pasteur) for Western blotting. The rest (6 serum samples out of the 55) showed one or two positive bands with only one of the two kits, but not with both kits. Therefore, these positive bands for the six serum samples appeared to be false. By the immune complex transfer enzyme immunoassay using each of the three antigens, all the 25 (19 plus 6) serum samples were negative. Thus the specificities of the immune complex transfer enzyme immunoassays and Western blotting were 100% (25/25) and 80% (20/25)—96% (24/25), respectively.

Thirty-two serum samples containing rheumatoid factor (13 samples) and autoantibodies to thyroglobulin (6 samples), DNA (10 samples), and acetylcholine receptor (3 samples) from HIV-1 seronegative subjects were tested by the immune complex transfer enzyme immunoassay using each of the three antigens (19). The concentration of IgG in these serum samples was 21 ± 9.2 (SD) mg/ml (range, 7.5–59 mg/ml), and levels of autoantibodies to thyroglobulin in three serum samples out of the 32 and to DNA in one serum sample were too high to be quantified without dilution. All the serum samples showed lower signals than the highest signals for the 100 HIV-1 seronegative subjects shown in Figures 11 and 12. In addition, there was only very weak correlation between the signal and the concentration of IgG in serum (r=0.37 with rRT, r = 0.64 with rp24, and r = 0.30 with rp17), and little correlation was observed between signals with two antigens out of the three (r = 0.02 between signals with rRT and rp17, 0.08 between signals with rRT and rp24 and 0.24 between signals with rp17 and rp24). These results indicated that the concentration of immunoglobulins in serum samples was not a major cause for higher signals among seronegative samples, but that a cause(s) for higher signals among seronegative samples was different among the three antigens.

Detection of Seroconversion

Eleven HIV-1 seroconversion serum panels (SV-0111, SV-0s immunoassay using recombinant gp41 and p24 as antigens (Abbott Laboratories, North Chicago, IL) and the gelatin particle agglutination test using a lysate of HIV-1 as antigen (Fujirebio) (Table 5) (20). In nine seroconversion serum panels (Panels 1–9) out of the 11, antibody IgGs to p17, p24, and RT were detected by the immune complex transfer enzyme immunoassays as early as or even earlier (Panel 3) than antibodies to HIV-1 by the other methods. In one of the 11 (Panel 10), antibody IgG to p17 was detected by the immune complex transfer enzyme immunoassay as early as antibodies to HIV-1 by the other methods, but antibody IgGs to p24 and RT were detected later. In another panel (Panel 11), the detection of antibody IgG to p17 by the immune complex transfer enzyme immunoassay was as early as the detection of antibodies to HIV-1 by the gelatin particle agglutination test, but was later than the detection of antibodies to HIV-1 by the third generation enzyme immunoassay and the detection of antibody IgGs to p24 and RT by the immune complex transfer enzyme immunoassay was later.

In previous reports, the earliest positive band by Western blotting after HIV-1 infection has been reported to be for p24 in some cases (46–48), but for *env* proteins in other cases (49–51). Therefore, the immune complex transfer enzyme immunoassay for antibody IgG to *env* proteins may be useful for detecting seroconversion very early after the infection.

TABLE 4. Specificity of Various Methods

Serum group[a]	Test results (positive number/tested number) ELISA	GPA[b]	Sample no. of serum	Western blotting[c] gp 160	gp 120	p 51	gp 41	p 31	p 24	p 17	Immune complex transfer enzyme immunoassay rRT	rp17	rp24
							With Ortho kis						
I	0/50	0/50	1	–	–	–	–	–	+	–	0/50	0/50	0/50
			2	–	–	–	–	–	–	+			
			3–50	–	–	–	–	–	–	–			
							With Sanofi kit						
			1–2	–	–	–	–	–	–	–			
			3	+	–	–	–	–	–	–			
			4	–	–	+	–	–	–	+			
			5	–	–	–	+	–	–	–			
			6	–	–	–	+	–	–	–			
			7	–	–	–	–	+	–	–			
			8	–	–	–	–	–	+	–			
			9	–	–	–	–	–	+	–			
			10	–	–	–	–	–	+	–			
			11	–	–	–	–	–	+	–			
			12	–	–	–	–	–	–	+			
			13	–	–	–	–	–	–	+			
			14	–	–	–	–	–	–	+			
			15–50	–	–	–	–	–	–	–			
							With Ortho kit						
II	25/25	2/25 (serum nos. 53 and 56)	51	–	+	–	+	–	–	–	0/25	0/25	0/25
			52–75	–	–	–	–	–	–	–			
							With Sanofi kit						
			51	–	–	–	–	–	–	–			
			52	–	–	–	–	–	+	+			
			53	–	–	–	–	–	+	–			
			54	–	–	–	–	–	+	–			
			55	–	–	–	–	–	+	–			
			56	–	–	–	–	–	–	+			
			57–73	–	–	–	–	–	–	–			

[a]Group I was from healthy subjects with low risk for HIV infection; group II comprised 25 sera, which were positive by the conventional ELISA but negative or showed one or two positive bands by Western blotting.
[b]Gelatin particle agglutination test.
[c]For Western blotting, only positive bands are shown, and negative results for p66 and p55 bands are not shown.

DETECTION OF p24 ANTIGEN IN SERUM

Immune Complex Transfer Enzyme Immunoassay for p24 Antigen

An ultrasensitive immunoassay (two-site immune complex transfer enzyme immunoassay) for p24 antigen of HIV-1 has been developed by applying the same principle as used for the detection of antibodies as shown in Figure 13 (22). The antigen was reacted simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-anti-rp24 Fab′ conjugate and anti-rp24 Fab′-β-D-galactosidase conjugate. The complex formed, comprising the three components, was trapped onto polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The polystyrene beads were washed to eliminate excess of anti-rp24 Fab′-β-D-galactosidase conjugate. The complex was eluted from the polystyrene beads with excess of ε*N*-2,4-dinitrophenyl-L-lysine and was transferred onto polystyrene beads coated with streptavidin to eliminate nonspecifically bound anti-rp24 Fab′-β-D-galactosidase conjugate more completely. β-D-Galactosidase activity bound to the last beads was assayed by fluorometry.

Detection Limit and Assay Range of p24 Antigen

The detection limit of rp24 per assay is 0.1 amol (2.4 fg) and 30-fold smaller than that by the conventional two-site enzyme immunoassay using polystyrene beads coated with anti-rp24 IgG and anti-rp24 Fab′-β-D-galactosidase conjugate. The assay range of p24 in serum is 0.24–720 pg/ml, since the signal increased linearly with up to 7.2 pg/tube and 10 μl of serum can be used without serum interference (22).

TABLE 5. Test Results of Seroconversion Serum Panels by Various Methods[a]

No. of seroconversion serum panels	Day of blood collection	Cutoff index by immune complex transfer enzyme immunoassay[b]					Cutoff index by conventional EIA (HIV-1/HIV-2 3rd generation, Abbott)	Maximal dilution to cause gelatin particle agglutination (Fujirebio)	Western blot (Ortho)[c]					
		Anti-p17	Anti-p24	Anti-RT	p24 antigen	Simultaneous detection			gp160	p66	p55	p51	p24	p17
1	0	0.2	0.1	0.2	0.3	0.9	0.3	–	–	–	–	–	–	–
(Panel E)	49	0.7	0.2	0.1	0.5	0.6	0.2	–	–	–	–	–	–	–
	63	0.4	0.2	0.1	0.5	1.1	0.2	–	–	–	–	–	–	–
	84	0.6	0.1	0.2	25	9.4	0.2	–	–	–	–	–	–	–
	91	0.4	0.1	0.2	8092	2793	0.8	–	–	–	–	–	–	–
	126	9786	258	20	14	4020	4.6	32	+	–	–	–	+	+
2	0	0.5	0.1	0.1	0.5	1.1	0.3	–	–	–	–	–	–	–
(Panel J)	14	0.4	0.1	0.2	163	40	0.4	–	–	–	–	–	–	–
	26	2807	104	1988	3.8	2951	2.3	32	+	+	–	+	+	–
	28	3589	127	2110	2.2	3530	2.3	32	+	+	–	+	+	–
	32	4897	231	3628	1.3	4518	2.8	32	+	+	–	+	+	–
	35	5458	236	3575	1.2	5365	2.9	32	+	+	–	+	+	–
	40	7415	320	5742	0.9	8117	3.7	32	+	+	–	+	+	–
3	**0**	**0.0**	**0.1**	**0.1**	**0.3**	**0.9**	**0.3**	–	–	–	–	–	–	–
(Panel K)	**6**	**0.1**	**0.1**	**0.1**	**0.5**	**0.8**	**0.3**	–	–	–	–	–	–	–
	8	**0.8**	**0.5**	**0.1**	**0.5**	**4.7**	**0.4**	–	–	–	–	–	–	–
	13	**364**	**11**	**0.6**	**0.7**	**153**	**0.7**	–	–	–	–	–	+	–
	20	**3232**	**367**	**12**	**0.4**	**1369**	**1.4**	1	–	–	–	–	+	–
	29	**4805**	**693**	**183**	**0.5**	**1962**	**1.4**	2	+	–	–	–	+	–
	33	**6316**	**1284**	**591**	**0.3**	**3100**	**2.1**	4	+	+	–	+	+	+
	36	**6211**	**1212**	**702**	**0.4**	**3655**	**2.7**	4	+	+	–	+	+	+
4	0	0.0	0.0	1.5	0.4	1.3	0.2	–	–	–	–	–	–	–
(Panel P)	4	0.0	0.0	0.9	0.4	0.8	0.2	–	–	–	–	–	–	–
	9	0.0	0.0	1.4	0.7	1.2	0.3	–	–	–	–	–	–	–
	15	0.1	0.0	1.5	341	159	0.4	–	–	–	–	–	–	–
	30	2816	365	163	4.6	1037	2.9	32	+	–	–	–	+	–
	35	4142	470	459	2.7	2462	2.9	32	+	+	–	–	+	–
5	0	0.3	0.2	0.3	37	16	0.3	–	–	–	–	–	–	–
(Panel S)	9	103	25	1489	8.1	1097	4.2	8	–	–	–	–	+	–
	11	388	48	665	3.2	976	3.5	8	+	–	–	–	+	–
6	0	0.2	0.1	0.9	0.5	1.2	0.2	–	–	–	–	–	–	–
(Panel Z)	2	0.1	0.1	0.5	0.6	0.9	0.2	–	–	–	–	–	–	–
	7	0.1	0.1	0.5	13	4.9	0.3	–	–	–	–	–	–	–
	9	0.5	0.0	0.5	103	44	0.3	–	–	–	–	–	–	–
	27	968	399	66	4.9	317	4.7	16	+	–	–	–	+	–
	32	1848	448	54	3.7	621	3.4	16	+	–	–	–	+	–

(*continued*)

TABLE 5. Test Results of Seroconversion Serum Panels by Various Methods (*continued*)

No. of seroconversion serum panels	Day of blood collection	Cutoff index by immune complex transfer enzyme immunoassay[b]					Cutoff index by conventional EIA (HIV-1/HIV-2 3rd generation, Abbott)	Maximal dilution to cause gelatin particle agglutination (Fujirebio)	Western blot (Ortho)[c]					
		Anti-p17	Anti-p24	Anti-RT	p24 antigen	Simultaneous detection			gp160	p66	p55	p51	p24	p17
7	0	0.7	0.4	0.4	12	5.4	0.2	–	–	–	–	–	–	–
(SV-0161)	4	0.2	0.1	0.4	38	15	0.2	–	–	–	–	–	–	–
	7	1.3	0.1	0.8	67	36	0.5	–	–	–	–	–	–	–
	11	89	11	2.7	133	98	5.2	8	–	–	–	–	+	–
	15	1537	184	8.9	4.0	503	2.8	16	–	–	–	–	+	–
	18	2516	253	7.7	1.4	667	2.1	16	–	–	–	–	+	–
8	0	0.1	0.1	0.2	11	4.5	0.2	–	–	–	–	–	–	–
(SV-0211)	2	0.3	0.1	0.4	58	26	0.4	–	–	–	–	–	–	–
	13	8367	46	48	107	1610	6.3	4	+	–	–	–	+	–
	15	10239	266	191	65	2164	6.2	16	+	–	–	–	+	–
	20	11302	660	1010	11	2280	3.7	32	+	–	–	–	+	–
	22	10749	662	1636	8.5	2680	3.3	32	+	–	–	–	+	–
9	0	0.5	0.3	1.1	0.7	1.8	0.2	–	–	–	–	–	–	–
(SV-0241)	6	0.8	0.6	2.0	77	37	0.2	–	–	–	–	–	–	–
	8	0.3	0.1	0.7	31	13	0.3	–	–	–	–	–	–	–
	14	42	4.2	9.1	3.1	33	7.4	8	+	–	–	–	+	–
	16	433	34	65	1.6	232	6.0	16	+	–	–	–	+	–
	21	3554	319	471	1.2	1292	5.6	16	+	–	–	–	+	–
	23	5454	396	580	0.7	1635	4.2	16	+	–	–	–	+	–
10	0	1.5	0.3	0.5	366	154	0.3	–	–	–	–	–	–	–
(SV-0111)	1	1.8	0.5	0.8	559	249	0.5	–	–	–	–	–	–	–
	7	3.6	0.5	0.3	124	54	4.8	32	–	–	–	–	+	–
	15	4716	370	18	16	1876	3.7	64	+	–	+	–	+	–
	19	5142	307	15	5.7	1945	3.0	64	+	–	+	–	+	–
	21	5368	298	21	6.1	1736	3.2	64	+	–	+	–	+	–
	26	6316	267	58	3.3	1939	3.3	64	+	–	+	–	+	–
11	0	0.3	0.1	0.2	17	12	0.4	–	–	–	–	–	–	–
(SV-0051)	6	0.2	0.1	0.2	232	87	1.4	–	–	–	–	–	–	–
	8	2.5	0.2	0.5	97	31	4.4	1	–	–	–	–	–	–
	15	6526	265	169	14	2055	4.0	8	+	–	–	–	+	–
	27	8632	858	914	1.3	2840	3.5	8	+	–	–	–	+	–

[a]Test results, except for those by the gelatin particle agglutination test and Western blotting, were expressed as the cutoff indices, i.e., the ratios of signals for serum samples to the cutoff value, which was the highest signal among 100 serum samples from seronegative subjects (65 males aged 24–68 yr and 35 females aged 35–68 yr). The highest signals among 100 seronegative subjects were 0.9, 1.9, 4.3, 4.4, and 1.9 in the immune complex transfer enzyme immunoassays for p24 antigen, antibody IgGs to p17, p24, and RT and the simultaneous detection, respectively.

[b]Immune complex transfer enzyme immunoassays were performed as described in Figures 11 and 15. The assay of p24 antigen and simultaneous detection were performed using monoclonal mouse anti-p24 Fab′-β-D-galactosidase conjugate. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr.

[c]No positive bands were observed for gp120, gp41, and p31 in Western blotting.

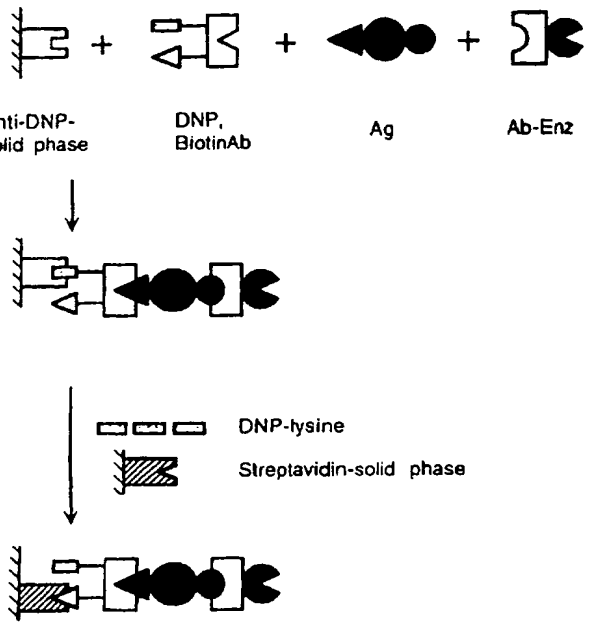

Fig. 13. Immune complex transfer enzyme immunoassay for antigen. DNP: 2,4-dinitrophenyl group. Ab: antibody. Ag: antigen. Enz: enzyme.

Detection of p24 Antigen in Serum Samples from HIV-1 Seropositive Subjects

Serum samples were collected from 79 HIV-1 seropositive subjects aged 10–61 yr—50 asymptomatic carriers, 9 patients with AIDS-related complex (ARC), and 20 patients with AIDS—and 117 HIV-1 seronegative subjects aged 24–68 yr.

Without acid treatment of serum samples, p24 antigen was detected in 24 serum samples (48%) out of 50 from the asymptomatic carriers, 7 serum samples (78%) out of 9 from the patients with ARC, 18 serum samples (90%) out of 20 from the patients with AIDS, and 6 serum samples (5%) out of 117 from the seronegative subjects.

When serum samples were treated at low pH to inactivate antibodies to HIV-1 p24 antigen with less impairment of p24 immunoreactivity (17,52,53), serum p24 levels were enhanced 1.4–17.4-fold in 19 serum samples (1.4–2.0-fold in 5 and >2.0-fold in 14) out of 50 serum samples from the asymptomatic carriers, 1.3–24-fold in 6 serum samples (1.3-fold in one and >2.0-fold in 5) out of 9 serum samples from the patients with ARC and 1.4–30.7-fold in 11 serum samples (1.4–2.0-fold in 3 and >2.0-fold in 8) out of 20 serum samples from the patients with AIDS. As a result, p24 antigen was detected in 34 serum samples (68%) out of 50 from the asymptomatic carriers, in 7 serum samples (78%) out of 9 from the patients with ARC, in 18 serum samples (90%) out of 20 from the patients with AIDS, and in none of 117 serum samples from the seronegative subjects.

Thus the specificity of the immune complex transfer enzyme immunoassay for p24 antigen in serum samples was 95% without acid treatment of serum, but was improved to 100% by acid treatment. Recently, the specificity even without acid treatment of serum has been improved to 100% by substituting monoclonal mouse anti-p24 Fab′-β-D-galactosidase conjugate for rabbit anti-p24 Fab′-β-D-galactosidase conjugate (54). The sensitivity for the asymptomatic carriers was 48% without acid treatment and was improved to 68% by acid treatment. The sensitivities for the patients with ARC and AIDS were 78% and 90%, respectively, regardless of acid treatment, although serum p24 levels in 55–67% of the samples were significantly enhanced by acid treatment as described above.

Detection of p24 Antigen in Seroconversion Serum Panels

Serum samples of 11 HIV-1 seroconversion serum panels (Panels 1–11), tested by the immune complex transfer enzyme immunoassays for antibody IgGs to p17, p24, and RT of HIV-1 and other methods, were subjected to the immune complex transfer enzyme immunoassay for p24 antigen using monoclonal mouse anti-p24 Fab′-β-D-galactosidase conjugate (Table 5) (20). In 10 panels out of the 11, signals for p24 antigen became positive 6–42 days earlier than those by the conventional ELISA, the gelatin particle agglutination test and Western blotting for antibodies to HIV-1, but declined as levels of antibody IgG or antibodies to HIV-1 rose. In one panel (Panel 3), p24 antigen was not detected.

SHORTENING OF THE WINDOW PERIOD

Choice of Antigens for Detection of Antibody IgG to HIV-1

The results described above for urine, whole saliva, and serum samples have indicated advantages of using rRT or rp66 of HIV-1 as antigen over other antigens for detection of antibodies to HIV-1 in HIV-1 seropositive subjects. By both the conventional ELISA (55) and Western blotting (56), the positive rates for antibodies to HIV-1 with rRT as antigen (100% in asymptomatic carriers, 86–95% in patients with ARC and 77–100% in patients with AIDS), were as high as those with *env* proteins as antigens (100% in asymptomatic carriers, 99–100% in patients with ARC, and 81–100% in patients with AIDS) and were higher than those with p17 as antigen (41% in asymptomatic carriers, 30% in patients with ARC, and 14% in patients with AIDS) and p24 as antigen (63% in asymptomatic carriers, 50–97% in patients with ARC, and 49–77% in patients with AIDS). By a sandwich enzyme immunoassay using rRT-coated microplates and rRT-alkaline phosphatase conjugate, seroconversion was detected as early as by the conventional ELISA using five recombinant proteins (gp120, gp41, p24, p17, and p15) as antigens and the

gelatin particle agglutination test using a lysate of HIV-1 as antigen (7). By the immune complex transfer enzyme immunoassay, the ratios of the lowest signals for HIV-1 seropositive subjects to the highest signal for HIV-1 seronegative subjects were higher with rRT as antigen than those with rp17 and rp24 as antigens, whether urine, whole saliva, or serum samples were tested (Figs. 6, 9, and 11) (12,18,19). In addition, levels of antibody IgG to RT decreased to less extents in patients with AIDS than those to p17 and p24 (Figs. 6, 9, and 11) (12,18,19). This is also consistent with a previous report that the positive rates remained high for antibody IgGs to p66 (RT), p31 and gp41 even at later stages of the infection, but decreased significantly for antibody IgGs to p17, p24 and gp120 (56). Moreover, the reaction of rRT with serum samples from HIV-1 seropositive subjects appears to be fairly specific (7,12,41,57). By a sandwich enzyme immunoassay using rRT-coated microplates and rRT-alkaline phosphatase for anti-HIV-1 antibodies, no reaction was observed with serum samples from subjects infected with either HIV-2 or hepatitis B virus (7). By the immune complex transfer enzyme immunoassays using rRT, rp17, and rp24 as antigens, no significant reaction was observed with serum samples from HTLV-I-infected subjects (12). RT of HIV has been described to be antigenically distinct from those of HTLV-I and II (57). However, it should be noted that signals by the immune complex transfer enzyme immunoassay with rRT as antigen were lower than those with p17 as antigen in most of HIV-1 seroconversion serum panels tested (Table 5) (20).

The disadvantage of using rRT as antigen described above can be overcome by using rp17 as antigen. In most of HIV-1 seroconversion serum panels tested, signals by the immune complex transfer enzyme immunoassay using rp17 as antigen were higher than those using rRT and rp24 as antigens, and antibody IgG to p17 was detected as early as or even earlier than not only antibody IgGs to RT and p24 by the immune complex transfer enzyme immunoassay, but also antibody IgG or antibodies to HIV-1 by the conventional ELISA and the gelatin particle agglutination test (Table 5) (20). However, the ratios of the lowest signals for HIV-1 seropositive subjects to the highest signal for HIV-1 seronegative subjects by the immune complex transfer enzyme immunoassay with rp17 as antigen were lower than those with rRT as antigen in samples collected probably sufficiently long after HIV-1 infection (Figs. 6, 9, and 11) (12,18,19).

Thus the seropositivity of HIV-1 infected subjects can be detected from early stages through late stages of the infection by the immune complex transfer enzyme immunoassay using both rRT and rp17 as antigens.

Simultaneous Detection of Both p24 Antigen and Antibody IgGs to p17 and RT

On the basis of the above results, reports, and considerations, the immune complex transfer enzyme immunoassay for simultaneous detection of both p24 antigen and antibody IgGs to p17 and RT in a single assay tube has been developed (Fig. 14), and 11 HIV-1 seroconversion serum panels were tested (Table 5 and Fig. 15) (20,21). For detection of p24 antigen, monoclonal mouse anti-p24 Fab′-β-D-galactosidase conjugate was used. Signals by the simultaneous assay became positive 6–42 days earlier than those by conventional methods for antibodies to HIV-1 and remained strongly positive even after signals for p24 antigen alone declined, although the cutoff indices by the simultaneous assay were lower than those for p24 antigen alone or for antibody IgG to p17 alone. In addition, 79 serum samples randomly collected from HIV-1 seropositive subjects (50 asymptomatic carriers, 9 patients with ARC, and 20 patients with AIDS) were subjected to the simultaneous detection. The ratios of the lowest signals for the asymptomatic carriers and the patients with ARC and AIDS to the highest signal for 100 HIV-1 seronegative subjects were 69,000, 199,000, and 3,330, respectively.

Thus by the simultaneous detection of p24 antigen and antibody IgGs to p17 and RT with the immune complex transfer enzyme immunoassay, both as early a diagnosis of HIV-1 infection as the appearance of p24 antigen in the circulation to shorten "the window period" and as reliable a diagnosis of the infection as that by the detection of antibodies to HIV-1 from the time of seroconversion until late stages of the infection became possible.

IMPROVEMENT OF IMMUNE COMPLEX TRANSFER ENZYME IMMUNOASSAY

In the immune complex transfer enzyme immunoassay described above, polystyrene beads were transferred from test tubes to test tubes with tweezers, and tips of the tweezers had to be washed carefully after each transfer of polystyrene beads to eliminate the false-positivity due to

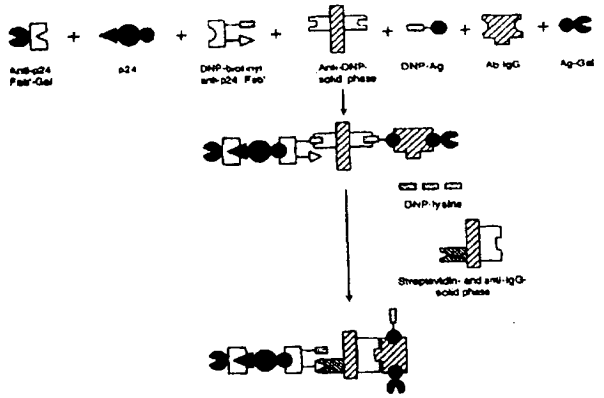

Fig. 14. Immune complex transfer enzyme immunoassay for simultaneous detection of both p24 antigen (left) and antibody IgG (right). DNP: 2,4-dinitrophenyl group. Ag: antigen. Ab: antibody. Gal: β-D-galactosidase.

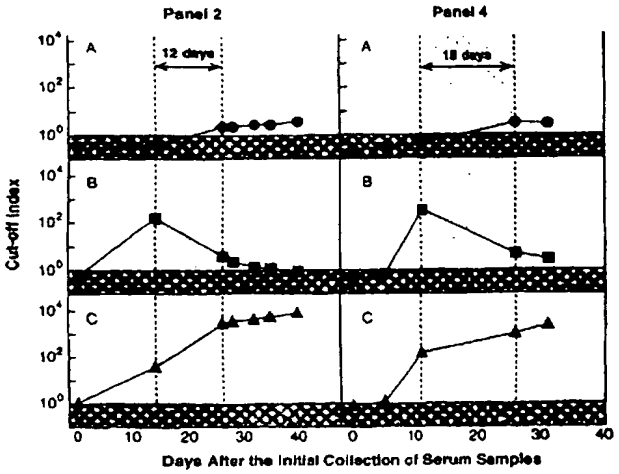

Fig. 15. Typical test results of seroconversion serum panels by the simultaneous detection shown in Figure 14. Serum samples (10 μl) of seroconversion serum panels were tested by the immune complex transfer enzyme immunoassay for simultaneous detection of both p24 antigen and antibody IgGs to RT and p17 using β-D-galactosidase from *E. coli* as label. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 hr. **A.** Abbott 3rd generation ELISA. **B.** Immune complex transfer enzyme immunoassay for p24 antigen. C. Simultaneous detection. Cutoff indices were calculated as shown in Table 5.

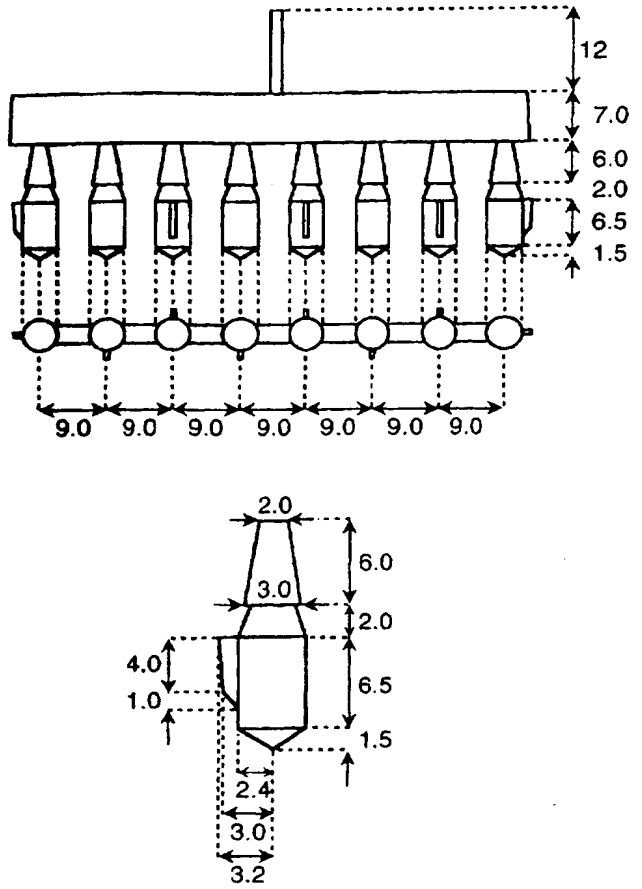

Fig. 16. Polystyrene stick for easy handling. Values indicate lengths in mm of various parts of polystyrene stick.

carryover (58). In addition, fluorescence intensities for bound enzyme activities of many samples were measured one by one with a spectrofluorophotometer. This was tedious and time-consuming, making the assay of many samples difficult.

These drawbacks have been recently minimized by substituting microplates, a fluororeader, and polystyrene sticks (Fig. 16) for test tubes, a spectrofluorophotometer, and polystyrene beads, respectively (Fig. 17) (59–61). Polystyrene sticks (Fig. 16) were transferred easily and quickly from wells to wells without using tweezers, eliminating false-positivity due to carryover. Fluorescence intensities of bound β-D-galactosidase activities for 96 samples could be measured within 1 min and 40 sec with a fluororeader. In addition, the sensitivity was also improved. Thus many samples could be tested using polystyrene sticks, microplates, and fluororeader much more easily with higher sensitivity and higher reliability.

Another drawback of the immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 described above is requiring a long time for its performance. This has been also recently overcome (62). The immune complex comprising 2,4-dinitrophenyl-bovine serum albumin-HIV-1 antigen conjugate, anti-HIV-1 IgG, and HIV-1 antigen-β-D-galactosidase conjugate was trapped onto polystyrene beads coated with (anti-2,4-dinitrophenyl group) IgG by overnight incubation and was transferred to polystyrene beads coated with (antihuman IgG γ-chain) IgG by 3 hr incubation in the presence of excess of εN-2,4-dinitrophenyl-L-lysine. These processes were made efficient by incubation with shaking and by using solid phases with larger surface areas. In addition, the volume of serum samples used was increased from 10 μl to 100 μl. As a result, the sensitivity was improved 20–30-fold, even when both trapping and transferring of the immune complex were performed for only 30 min. Furthermore, testing many samples became easily possible with higher sensitivity using microplates and a fluororeader.

With this improved immune complex transfer enzyme immunoassay for antibody IgG to p17 of HIV-1, 12 seroconversion panels were tested and antibody IgG to p17 was detected earlier than p24 antigen in four seroconversion serum panels of the 12 (to be published in detail elsewhere).

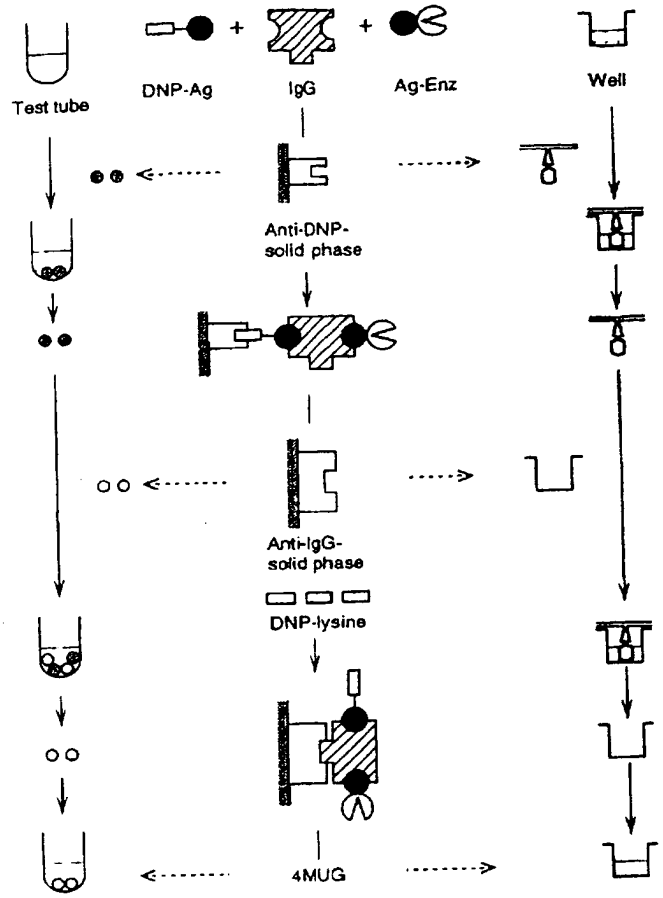

Fig. 17. Immune complex transfer enzyme immunoassays for antibody IgG using polystyrene beads in combination with test tubes and spectrofluorophotometer (left) and polystyrene sticks in combination with microplate wells and fluororeader (right). DNP: 2,4-dinitrophenyl group. Ag: antigen. Enz: enzyme. 4MUG:4-methylumbelliferyl-β-D-galactoside.

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